Homologous recombination (HR) is a highly conserved cellular process involved in the maintenance of chromosomal integrity and generation of genetic diversity. Biochemical and genetic studies have suggested that HR is crucial for repair of damaged DNA arising from various endogenous or exogenous assaults on the genome of any organism. Further, HR is vital to repair fatal DNA damage during DNA replication. An instructive example of cross-talk between the processes of DNA recombination and replication can be construed in the processing of replication/recombination/repair intermediates. The impediment(s) to the progression of DNA replication fork is one of the underlying causes for increased genome instability and consequently this might compromise the survival of organism. Various processes manifest at stalled replication forks before they can be rendered competent for the replication-restart. One of the mechanisms of replication-restart involves replication fork reversal (RFR), which envisage unwinding of the blocked forks with simultaneous annealing of the parental and daughter strands o generate a Holliday junction intermediate adjacent to DNA double strand end. Genetic evidence shows that in *E. coli dnaEts* mutant, *holD* mutant and in helicase defective *rep* mutant, RFR is catalyzed by RuvAB complex. Classically, HJ intermediates are generated during the terminal stages of the HR pathway. In *E. coli,* branch migration and resolution of HJ intermediates is promoted by RuvA, RuvB and RuvC proteins, which participate at the late stages of HR. Structural, biochemical and mutational analysis suggest that *E. coli* RuvA binds Holliday junction DNA with high affinity and specificity. RuvB, a member of the AAA^+ (ATPase associated with various cellular activities) family, is recruited to the RuvA-Holliday junction complex and functions as a motor protein. Together, RuvA and RuvB catalyze ATP dependent branch migration of HJ. The resolution of HJ is catalyzed by the RuvC endonuclease, which introduces coordinated cuts at two symmetrical sites across the junction.

RuvAB complex, the Holliday junction branch migration apparatus, is ubiquitous in bacteria. Genetic, biochemical and structural studies have not only established the in vivo role of *E. coli* RuvAB, in context of HR pathway, but have also provided valuable insights into the mechanism of HJ processing by RuvAB complex. However, the paucity of extensive studies examining the biochemical properties of each member of the RuvABC protein complex restricts models in deciphering the functions of the individual

components of this tripartite protein complex. Our current understanding of the biochemical function of E. coli RuvA is within the context of its interacting cellular partner, RuvB. Consequently, the inherent activities of RuvA in the context of DNA repair and HR are poorly understood. Moreover, it remains to be ascertained if RuvABC protein complex, its different sub-complexes, or the individual subunits can function differently in the processing of HJ intermediates generated during DNA repair and HR. The information from these studies would be helpful in understanding the mechanistic details of HR pathway in mycobacteria. Additionally, a number of important questions regarding the molecular basis of RuvAB catalyzed fork reversal remain unanswered. Therefore, exploration of biochemical details of the RuvAB mediated RFR would provide mechanistic insights into the dynamics of fork reversal process. Moreover, analysis of RuvAB catalyzed RFR might be helpful in validating the different assumptions of the RFR model that has been proposed on the basis of genetic analysis of certain E. coli replication mutants. Another interesting question that remains to be answered is, how under in vivo conditions, RuvABC protein complex or its individual subunits are regulated to function differently in the context of HR and DNA repair?

Mycobacterium tuberculosis is an important intracellular pathogen which is likely to experience substantial DNA damage inside the host and thus may require an efficient DNA recombination and repair machinery for its survival. Our knowledge about the mechanistic aspects of genetic exchange in mycobacteria is rather limited. Therefore, understanding of the processes catalyzed by the components of HR pathway may help in molecular genetic analysis of mycobacteria. Sequence analysis of M. tuberculosis genome, followed by various comparative genomic studies, has revealed the presence of putative homologs of E. coli rec genes but it is not known whether these gene products are able to catalyze the reactions similar to their *E. coli* counterparts. In *M. tuberculosis,* the genes encoding for the enzymatic machinery required for branch migration and resolution of HJ intermediates are present. The *ruvA, ruvB and ruvC* genes form an operon, and are probably translationally coupled. Further, these *ruv* genes are DNA damage inducible. The transcript level of *ruvC* is regulated by both RecA dependent and independent mechanisms whereas ruvA and ruvB are induced only through RecA

dependent SOS response. During *M. tuberculosis* infection of host cells, expression of ruvA and ruvB genes is upregulated. We therefore surmise that their gene product might be required for DNA replication, recombination or repair, and would be physiologically relevant under *in vivo* conditions. However, the details of reactions involved in the processing of HR intermediates and rescue of stalled replication forks in *M. tuberculosis* remains unknown.

In the initial part of this study, we have investigated the function of *M. tuberculosis* RuvA protein using Holliday junctions containing either homologous or heterologous core. In the later part, we have explored the ability of *M. tuberculosis* RuvA and RuvB proteins to catalyze *in vitro* replication fork reversal.

M. tuberculosis ruvA gene was isolated by PCR amplification and cloned in an expression vector to generate the *pMTRA* construct. Genetic complementation assays, using the *pMTRA* construct transformed into *E. coli ΔruvA* mutant, indicated that *M. tuberculosis ruvA* is functional in *E. coli* and suggested that it can substitute for *E. coli RuvA* in conferring resistance to MMS and survival following UV irradiation. Having established the functionality of *M.tuberculosis ruvA*, a method was developed for heterologous over-expression and purification of *M. tuberculosis RuvA* protein (MtRuvA). MtRuvA was purified to homogeneity and the identity of purified protein was verified using western blot analysis using the anti-MtRuvA antibodies. Purified MtRuvA was free of any contaminating endo- or exo-nuclease activity. Biochemical functions of MtRuvA were defined by performing detailed investigations of DNA-binding and Holliday junction processing activities. Substrate specificity of purified MtRuvA was examined,through DNA binding assays, by using oligonucleotide substrates mimicking differentintermediates involved in the pathway of recombinational DNA repair. Purified *M. tuberculosis* RuvA exhibited high affinity for HJ substrate but also formed stable complex with replication fork and flap substrate. DNase I footprinting of MtRuvA-homologous Holliday junction complex confirmed that MtRuvA bound at the junction center. The DNase I protection conferred by MtRuvA, on homologous HJ, was two-fold symmetric; the continuous footprint was 10 bp longon one pair of symmetrical arms and

7 bp on the opposite pair of arms. In parallel, DNase footprinting of MtRuvA-heterologous Holliday junction complex generated a footprint that encompassed 16 nucleotide residues on each strand of the Holliday junction. Different crystallographic studies have envisaged an important role for RuvA in base pair rearrangement atthe center of the junction. Also, in crystal structure of tetramer of EcRuvA-HJ complex twobases at the junction center were unpaired. To explore if RuvA binding leads to helical distortionof Holliday junction, MtRuvA-HJ complexes were subjected to chemical probing with KMnO4.In case of heterologous HJ, binding of MtRuvA resulted in appearance of sensitive T residues at the junction crossover. By contrast, binding of MtRuvA to homologous HJ rendered the T residues at the junction center and within the homologous core sensitive to oxidation by KMnO4.Taken together, these observations suggested that binding of MtRuvA distorts two base pairs at the junction crossover in heterologous HJ, whereas in case of homologous HJ base pairs distortion extends into the arms of the junction. These observations with KMnO4 probing were independently validated, in real time, by using sensitive to 2-aminopurine fluorescence spectroscopy measurements of MtRuvA-HJ complexes. To follow structural distortions upon interaction with MtRuvA, HJ variants carrying 2-AP substitution were generated for both homologous and heterologous HJ substrate. In each junction species, the 2-AP residue was uniquely present either at the junction center, adjacent to the center or away from the center. Incase of heterologous HJ, binding of MtRuvA resulted in increase of fluorescence emission of2-AP residues located at the junction crossover but not those of 2-AP residues that were present1-2 base pairs away from the junction center. Binding of MtRuvA to homologous HJ resulted in increase of fluorescence emission of 2-AP residues located at the junction crossover. Further, increase in fluorescence emission was also observed for 2-AP residues present within the homologous core or adjacent to the homologous core in a pair of symmetrically related arms. Thus, 2-AP fluorescence results suggested that binding of MtRuvA to homologous HJ causes base pair distortion within and adjacent to the homologous core whereas in case of heterologous HJ the base pair distortion is restricted to the junction center. Together, these results suggest thatMtRuvA causes two distinct types of base pair distortions between homologous and heterologous HJ substrates. To explore the relationship between binding of MtRuvA and alterations in

global structure of the junction DNA, we employed the established technique of comparative gel electrophoresis. Analysis of data from comparative gel electrophoresis revealed that MtRuvA, upon binding to the Holliday junctions, converts the stacked-X structure of HJ to square-planar form and stabilizes the same for loading of RuvB rings and subsequent branch migration by RuvAB complex. Our results underline the possible existence of distinct pathways for RuvA function, which presumably depend on the structure and the nature of the DNA repair or HR intermediates. In summary, our results show that binding of MtRuvA to the HJ induced changes in the local conformation of junction, which might augment RuvB catalyzed branch migration. An unexpected finding is the observation that MtRuvA causes two distinct types of structural distortions, depending on whether the Holliday junction contains homologous or heterologous core. These observations support models wherein RuvA facilitates, in a manner independent of RuvB, base pair rearrangements at the crossover point of both homologous and heterologous Holliday junctions.

Although the genetic basis of *ruvA ruvB* catalyzed RFR in *E. coli* has been understood in some detail but less is known about the genetic and molecular mechanism of fork reversal in mycobacteria or other organisms. Specifically, to examine if the *E. coli* paradigm can be generalized to other RuvAB orthologs, we explored the RFR activity of M. tuberculosis RuvAB using a series of oligonucleotides and plasmid-based substrates that mimic stalled replication fork intermediates. This approach might be useful in genetic analysis of factors involved in processing of stalled forks in *M. tuberculosis* wherein technical difficulties associated with the isolation and characterization of appropriate mutants have limited our understanding of DNA metabolism. Importantly, we have asked the questions as to how the structure at fork junction, extent of reversal and presence of sequence heterology might determine the outcome of RuvAB mediated RFR. The results from this study will be helpful in consolidating the proposed *in vivo* role for RuvAB complex in fork reversal.

The open reading frame corresponding to *M. tuberculosis ruvB* gene was PCR amplified and cloned in an expression vector to generate the *pMTRB* construct. Genetic complementation assays were performed to assess the functionality of *M. tuberculosis*

ruvB in *E. coli ΔruvB* mutant. The data from these assays suggested that *M. tuberculosis ruvB* is active in E. coli and it is able to make functional contacts with *E. coli RuvA.* Moreover, the efficient alleviation of MMS toxicity in *E. coli ΔruvB* mutant suggested that *M. tuberculosis ruvB* might have a role in relieving replication stress generated under specific in vivo conditions. For biochemical analysis, *M. tuberculosis RuvB* protein (MtRuvB) was over-expressed in a heterologous system and purified to homogeneity. The identity of purified MtRuvB was verified using western blot analysis using the anti-MtRuvB antibodies. Purified MtRuvB was free of any contaminating endo- or exo-nuclease activity. The DNA-binding properties of MtRuvB were analyzed, in conjunction with its cognate RuvA, by using different substrates that are most likely to occur as intermediates during the processes of DNA replication and/or recombination.

MtRuvAB bound HJ, three-way junction and heterologous replication fork with high affinity but with relatively weaker affinity to flap and flayed duplex substrates. MtRuvB displayed very weak affinity for linear duplex and failed to bind linear single-stranded DNA. The high affinity of MtRuvB for HJ substrate, in presence of its cognate RuvA, is indicative of direct and functional interaction between RuvA and RuvB. To further test this idea, the catalytic activity of MtRuvB was assayed in the *in vitro* HJ branch migration assay. In this assay,MtRuvB, in association with its cognate RuvA, promoted efficient branch migration of homologous HJ over heterologous HJ. To decipher the role of MtRuvAB in processing of stalled replication fork we performed in vitro replication fork reversal (RFR) assay using both oligonucleotide and plasmid based model replication fork substrates. Initially, binding of MtRuvAB to different homologous fork (HomFork) substrates was analyzed using the electrophoretic mobility shift assays. MtRuvAB exhibited similar binding affinity towards different HomFork substrates bearing different spatial orientation of nascent leading and lagging strands. To gain insight into the role of MtRuvAB in processing of replication forks, *in vitro* RFR reactions were carried out using an array of synthetic homologous fork substrates. In all these reactions, MtRuvAB catalyzed efficient fork reversal leading to generation of both parental duplex and daughter duplex. In the kinetics of fork reversal reaction, for all the fork substrates,the accumulation of daughter duplex increased with time whereas the

increase in parental or nascent strand DNA was negligible. Taken together, our results suggest that MtRuvAB can efficiently catalyze in vitro replication fork reversal reaction to generate a Holliday junction intermediate thus implicating that RuvAB mediated fork reversal involves concerted unwinding and annealing of nascent leading and lagging strands. Equally important, we demonstrate the reversal of forks carrying hemi-replicated DNA, thus indicating that MtRuvAB mediated fork reversal is independent of symmetry at the fork junction. For understanding the role of RuvAB mediated processing of stalled forks at chromosome level, the fork reversal assays were performed using plasmid derived model "RF" substrate. Fork reversal was monitored by restriction enzyme digestion mediated release of 5' end labeled fragments of specific size from the fourth arm extruded at the branch point of fork junction. In these reactions MtRuvAB complex was proficient at generating the reversed arm *de novo* from the RF substrate. Further, MtRuvAB complex catalyzed extensive fork reversal as analyzed by release of linear duplex of2.9 kb from a JM substrate. Use of non hydrolysable analogs of ATP and analysis of restriction digestion mediated release of duplex fragments from the reversed arm suggested that MtRuvAB catalyzed RFR reaction is ATP hydrolysis dependent progressive and processive reaction. MtRuvAB complex catalyzed fork reversal on plasmid substrate that had been linearized thus indicating that MtRuvAB mediated RFR is uncoupled from DNA supercoils in the substrate. Notably, MtRuvAB promoted reversal of forks in a substrate containing short stretch of heterologous sequences, indicating that sequence heterology failed to impede fork reversal activity of MtRuvAB complex. These results are discussed in the context of recognition and processing of varied types of replication fork structures by RuvAB enzyme complex.